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The Latent Membrane Protein 1 of Epstein-Barr Virus Establishes an Antiviral State via Induction of Interferon-stimulated Genes

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Abstract

Epstein-Barr virus (EBV) infection is associated with several human cancers. Latent membrane protein 1 (LMP-1) is one of the key viral proteins required for transformation of primary B cells *in vitro* and establishment of EBV latency. In this report, we show that LMP-1 is able to induce the expression of several interferon (IFN)-stimulated genes (ISGs) with antiviral properties such as 2'-5' oligoadenylate synthetase (OAS), stimulated *trans*-acting factor of 50 kDa (STAF-50), and ISG-15. LMP-1 inhibits vesicular stomatitis virus (VSV) replication at low multiplicity of infection (0.1 pfu/cell). The antiviral effect of LMP-1 is associated with the ability of LMP-1 to induce ISGs; an LMP-1 mutant that cannot induce ISGs fails to induce an antiviral state. High levels of ISGs are expressed in EBV latency cells in which LMP-1 is expressed. EBV latency cells have antiviral activity that inhibits replication of superinfecting VSV. The antiviral activity of LMP-1 is apparently not related to IFN production in our experimental systems. In addition, EBV latency is responsive to viral superinfection: LMP-1 is induced and EBV latency is disrupted by EBV lytic replication during VSV superinfection of EBV latency cells. These data suggest that LMP-1 has antiviral effect, which may be an intrinsic part of EBV latency program to assist the establishment and/or maintenance of EBV latency.

Abbreviations: EBV, Epstein-Barr virus; CTAR, C-terminal activator region; IFN, interferon; HIV, human immunodeficiency virus; FBS, fetal bovine serum; VSV, vesicular stomatitis virus; pfu, plaque-forming unit; moi, multiplicity of infection; LMP, latent membrane protein 1; ISG, IFN-stimulated genes; IRF, IFN regulatory factor; RT, reverse transcription; OAS, oligoadenylate synthetase; STAT, signal transducer and activator of transcription.

Introduction

Epstein-Barr virus (EBV)¹ is a prototype of human γ -herpes-virus of increasing medical importance. EBV infection is an important cause of lymphomas in patients with advanced HIV infection or AIDS, and in severely immunocompromised people, especially organ transplant recipients. Also, EBV infection is associated with the development of nasopharyngeal carcinoma (NPC) and Burkitt's lymphoma (BL) (1, 2).

The biologic hallmark of the EBV-cell interaction is latency. Three types of latency have been described, each having its own distinct pattern of gene expression. Type I latency is exemplified by BL tumors *in vivo* and earlier passages of cultured cell lines derived from BL biopsies. EBV nuclear antigen 1 (EBNA1) protein is expressed in this form of latency. Type II latency is exemplified by NPC and

Hodgkin's disease. EBNA1, latent membrane protein 1 (LMP-1), LMP2A, and LMP2B proteins are expressed in type II latency. Type III latency is represented by lymphoblastoid cell lines (LCLs). Nine viral proteins are expressed, including six nuclear proteins (EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, and EBNA-LP) and three integral membrane proteins (LMP-1, LMP-2A, and LMP-2B) (reviewed in References 1 and 2).

LMP-1 is an essential gene required for EBV transformation and establishment of latency *in vitro*. LMP-1 is an integral membrane protein with six transmembrane-spanning domains in the N terminus and a C-terminal domain located in the cytoplasm (2, 3). Two C-terminal activator regions (CTARs) have been identified to initiate signal transduction. LMP-1 acts as a constitutively active receptor-like molecule that does not need the binding of a ligand (4). LMP-1 appears to be a central effector of altered cell growth, survival, adhesive, and invasive potential (5–10).

Interferons (IFNs) are cytokines with antiviral activity. The ability of IFNs to induce an antiviral state on cells is their fundamental property (11, 12). IFNs are produced upon the infection of cells by viruses. The mechanism of the transcriptional activation has been under intensive investigation. One of the major players in IFN production is IFN regulatory factor 7 (IRF-7). IRF-7 can be activated by phosphorylation and nuclear translocation upon viral infection and activated IRF-7 is partially responsible for transcriptional activation of IFNs (13–17).

IFNs bind to the receptor on cell surface and activate a cascade of intracellular signaling pathways leading to up-regulation of more than 1000 IFN-stimulated genes (ISGs) within the cell. STAT-1 is a major component of signal transducers for IFN for ISG production (reviewed in References 11 and 12).

ISGs have multiple functions. The induction of antiviral ISGs confers the cells with antiviral activity. The antiviral functions of several ISGs are well understood. The IFN-inducible 2'-5' oligoadenylate synthetase (OAS) catalyzes the synthesis of oligoadenylates of the general structure ppp(A2'p)nA, commonly abbreviated 2–5A. RNase L, a latent endoribonuclease, becomes activated by binding 2–5A oligoadenylates. Activated RNase L catalyzes the extensive cleavage of single-stranded RNA of both viral and cellular origins, including rRNA (11, 12). Overexpression of OAS alone is sufficient to block the replication of virus in the absence of any other IFN-inducible proteins (18).

Other well established antiviral ISGs are dsRNA-activated protein kinase, PKR, and myxovirus resistance-1 (Mx1 for mice and MxA for humans) genes. The antiviral effect of PKR is due to its phosphoryla-

tion of the alpha subunit of initiation factor (eIF-2 α). This phosphorylation results in rapid inhibition of translation. Mx proteins interfere with the growth of influenza and other negative strand RNA viruses at the level of viral transcription and at other steps (11, 12).

Many ISGs are poorly characterized, and some of these are very likely to possess antiviral activity. Stimulated *trans*-acting factor of 50 kDa (STAF-50) is a member of the ring finger family, now collectively called TRIM for proteins containing a tripartite motif (19). STAF-50 has a significant inhibition of the retroviral infections (20). It is also a target and down-regulated in human papillomavirus-infected cells (21). ISG-15 is involved in the repression of human immunodeficiency virus (HIV) replication by IFN (22). Also, expression of ISG-15 is correlated with antiviral responses (23, 24). Clearly, the enormous selective pressures imposed by viral infection have resulted in a rich and diverse set of antiviral pathways.

Previously, we have shown that LMP-1 induces the expression of IRF-7 and STAT-1; both of which are ISGs and involved in the antiviral response of cells (25, 26). In this report, we extend our earlier discovery and show that LMP-1 induces several other antiviral ISGs implicated in cellular antiviral responses. We further show that LMP-1 possesses antiviral activity, and EBV latency cells inhibit the replication of superinfecting viruses. We propose that the antiviral effect of LMP-1 may be an intrinsic part of EBV latency, which may be used to assist the maintenance and/or establishment of EBV latency.

Materials and Methods

Plasmids, Antibodies, and Viruses—Expression plasmids of LMP-1 and mutant (LMP-DM) were described previously (27). ISG-15 monoclonal antibody from Dr. Bordin; and MxA antibody from Dr. Mark A. McNiven (28). Tubulin was purchased from Sigma. STAT-1 antibody (sc-417, sc-591) was from Santa Cruz Biotechnology. Phospho-Tyr-701 STAT-1 antibody (06-657) was from Upstate Biotechnology. LMP-1 Ab (CS1-4) was purchased from Dako. Stock of VSV (Indiana serotype) was prepared and titrated in BHK-21 cells. A mouse polyclonal antibody raised against purified VSV was used for detection of VSV proteins in infected cells. Sendai virus stock was purchased from Spafas, Inc. Anti-sendai virus antibody was purchased from US Biologicals, Inc. Recombinant human IFN- α 2a was purchased from Hoffman-La Roche Inc.

Cell Culture, Transient Transfection, and Isolation of Transfected Cells—DG75 is an EBV-negative Burkitt's lymphoma cell line (29). BL41 is EBV-negative BL lines, BL41-EBV was generated by *in vitro* infection with EBV B95-8 strain (30). SavI, Daudi, P3HR-1, and Jijoye are all EBV-positive BL lines (31–33). These cells were maintained in RPMI 1640 plus 10% fetal bovine serum (FBS). Baby hamster kidney (BHK-21) cells are maintained in MEM containing 5% FBS. 293 are human fibroblasts and are maintained in Dulbecco's modified Eagle's medium plus 10% FBS. Electroporation was used for transfection of the B cells as described previously (26, 27, 34). Enrichment for CD-4-positive cells was performed with the use of anti-CD-4-antibody conjugated to magnetic beads according to the manufacturer's recommendation (Dynal, Inc.). DG75 cells were transfected with CD-4 expression and other plasmids. One day after the transfection, the cells were used for isolation of CD-4-positive cells with the use of Dynabeads CD4 (Dynal Inc.). The transfected cells were incubated with Dynabeads CD4 at 72 μ l of beads/10⁷ cells for 20–30 min at 4 °C with gentle rotation. CD4-positive cells were isolated by placing the test tube in a magnetic separation device (Dynal magnet). The supernatant were discarded while the CD4-positive cells are attached to the wall of the test tube. The CD4-positive cells were washed 4–5 times in phosphate-buffered saline plus 2% FBS, and resuspended in 100 μ l RPMI 1640 plus 1% FBS. Cells were detached from the Dynabeads CD4 by incubate for 45–60 min at room temperature with 10 μ l of DETACHaBEAD. The detached beads were removed by using a magnetic separation device. The released cells were washed 2–3 times with 500 μ l of RPMI 1640 plus 10% FBS, and resuspended in RPMI 1640 plus 10% FBS at 5 \times 10⁵ cells/ml. The isolated cells were used to extract total RNAs or prepare cell lysates immediately, or recovered overnight before infection by viruses.

Western Blot Analysis with Enhanced Chemiluminescence (ECL)—Separation of proteins on SDS-PAGE was carried out following standard protocol. After the proteins were transferred to a nitrocellulose or Immobilon membrane, the membrane was blocked with 5% nonfat dry milk in TBST (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.05% Tween-20) at room temperature for 10 min. It was then washed briefly with water, and incubated with the primary antibody in 5% milk in TBST for 1 h at room temperature, or overnight at 4 °C. After washing with TBST three times (10 min each), the membrane was incubated with the secondary antibody at room temperature for 1 h. It was then washed three times with TBST, treated with ECL detection reagents (Amersham Biosciences), and exposed to Kodak XAR-5 film.

RNA Extraction and RNase Protection Assays (RPA)—Total RNA was isolated from cells using the RNeasy Total RNA Isolation Kit (Qiagen, Valencia, CA). RPA was performed with total RNA using the RNase Protection Assay Kit II (Ambion, Houston, TX). The GAPDH probe was from US Biochemicals, Inc. The probe for STAF-50 was made from the PCR product amplified using the two primers from STAF-50: 5'-GGGGTACCGACGTCATGAAAGGAGTG-3' and 5'-GGATTGGAATTCTTAATGTG-3'. The PCR product was then cloned into pcDNA3 vector. The probe for OAS was a gift from Dr. Teresa Compton.

Plaque Assays—BHK-21 cells were grown in 12- or 6-well plates to 90% confluency. Cells were infected in duplicates with serial 10-fold dilutions of cell culture supernatants. After an initial adsorption of 45 min, the inoculum was aspirated out, and cells were washed two times with medium. Cells were then overlaid with 1.5 ml of MEM containing 2% FBS and 0.75% low melting point agarose (Invitrogen) and incubated for 15–18 h. After the incubation period, cells were fixed with 1 ml of 2% glutaraldehyde in phosphate-buffered saline for 1 h at room temperature. The agarose plugs were then removed, and cells were stained for 10–15 min with 0.01% crystal violet in 30% methanol. Cells were then washed with water and air-dried. The plaques were counted, and titers determined by taking the average.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Total RNA was isolated from samples by using RNeasy kit (Qiagen). The RNA samples were treated with DNase I at 37 °C for 30 min. The primers used in this experiment are as follows. For all IFN- α genes, the consensus primers for all IFN- α subtypes were used. IFNA5: 5'-AGA ATC TCT CCT TTC TCC TG-3' and IFNA3: 5'-TCT GAC AAC CTC CCA GGC AC-3'; For IFN- α 1: IFNA15, 5'-GCA ATA TCT ACG ATG GCC TC-3' and IFNA13, 5'-CAG AAT TTG TCT AGG AGG TC-3'; For actin gene: Actin1, 5'-TTC TAC AAT GAG CTG CGT GT-3' and Actin 2, 5'-GCC AGA CAG CAC GTT GTT GG-3'. Positive controls were RNAs from either 293 cells transfected with constitutively active form of IRF-7 (IRF7 Δ 247–467) (16) or 293 cells infected with 200 hemagglutinin units of Sendai virus. Both positive controls produced similar results. RT-PCR was performed with SuperScriptTM One-Step RT-PCR with Platinum[®] Taq (Invitrogen) following manufacturer's protocol. One set of primers for IFN and actin primers were mixed for the detection of IFN and actin simultaneously.

Statistical Analyses—Mean values \pm S.D. were calculated by Microsoft Excel program. The significance between two groups was calculated by Mann-Whitney U Test with the use of the Statistica 6.0 program.

Results

LMP-1 Selectively Stimulates the Expression of Antiviral ISGs—We have previously shown that LMP-1 induces IRF-7, STAT-1, and STAT-2 in human B cells (25, 26). In addition, EBV infection induces expression of ISGs (35). All these available data suggest that EBV, or LMP-1 in particular, may have the ability to induce other ISGs. Because of the role of STAT-1 and IRF-7 in cellular antiviral responses, we examined whether LMP-1 can induce other known antiviral ISG genes. DG75 cells, which are EBV-negative Burkitt's lymphoma cells, were used for the test because of the transfection efficiency. LMP-1 has two critical domains (CTAR-1 and CTAR-2) for its signaling. Point mutations at the CTAR-1 that change the PX-QXT motif into AXAXT will destroy the signaling pathways initiated from CTAR1. The tyrosines (Y) in the last three amino acids of LMP-1 (YYD) have been shown to play an important role in the signaling pathway of CTAR2; the mutations of YYD abolish the activation of NF- κ B and AP-1. LMP-DM has point mutations at the critical residues in both CTAR-1 and -2 (27). LMP-1 or LMP-DM, was

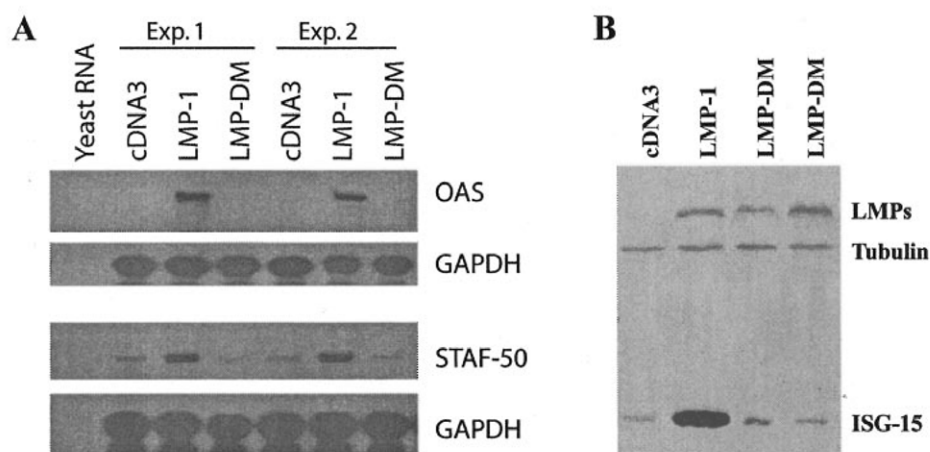


Figure 1. LMP-1 stimulates the expression of ISGs. A, LMP-1 induces expression of OAS and STAF-50 RNA. Total RNAs (5 μ g) from DG75 cells transfected with pcDNA3, LMP-1, or LMP-DM expression plasmids were used for RPA. OAS plus GAPDH, or STAF-50 plus GAPDH probes were used. Yeast RNA was used as negative control. Specific protections of OAS, STAF-50, and GAPDH RNAs are indicated. The relative level of STAF-50 RNA (STAF-50/GAPDH) induced by LMP-1 is 3.8-fold calculated from three independent experiments (two of which are shown). B, LMP-1 induces expression of ISG-15 protein. Lysates from pcDNA3, LMP-1, or LMP-DM expression plasmid transfected and enriched DG75 cells were used for Western blot analysis with tubulin, ISG15 as well as LMP-1 antibodies. The identity of proteins is as shown.

transfected into DG75 cells and the transfected cells were enriched by CD4 selection (see “Materials and Methods” for detail). Western blotting or RNase protection assays (RPAs) were used for detection of the expression of target genes based on availability of probes and specific antibodies. As shown in Figure 1A, LMP-1 induced the RNA expression of OAS and STAF-50. In addition, LMP-1 caused a marked increase in ISG-15 protein levels (Figure 1B). However, LMP-DM failed to induce the expression of OAS, STAF-50, and ISG15. The results indicate that LMP-1 induced expression of the three ISGs, and the two CTARs of LMP-1 were required for the induction. However, LMP-1 could not induce the expression of PKR, IRF-1, or MxA proteins in DG75 cells (data not shown). Thus, these data indicate that LMP-1 selectively induces antiviral ISGs.

LMP-1 Inhibits VSV Protein Expression—One of the common functions for OAS, IRF-7, STAT-1, STAF-50, and ISG-15 proteins is their potential roles in the establishment of antiviral state in a cell (for a review, see References 11 and 12). It is possible that LMP-1 might have an antiviral effect via the induction of these ISGs. To test the hypothesis, we first examined whether viral gene expression was affected in LMP-1-expressing cells. LMP-1 expression plasmid or its vector (pcDNA3) was transfected into DG75 cells, and the transfected cells were selected for vesicular stomatitis virus (VSV) infection for 8–10 h. The reason to use VSV is mainly because VSV infects human B cells efficiently. Figure 2 shows the comparison between LMP-1-expressing cells and vector control cells in their abilities to support VSV gene expression. At high multiplicity of infection (moi), LMP-1 could not inhibit the expression of VSV proteins effectively (lanes 3 and 4). However at lower moi (0.1 pfu/cell), LMP-1 significantly inhibited VSV protein expression (lanes 5 and 6). Also, IFN repressed VSV protein expression as expected (data not shown). In addition, second non-related virus, sendai virus, was used to infect LMP-1-expressing cells. LMP-1 also inhibited the sendai virus gene expression (data not shown). Thus, LMP-1 was able to repress VSV gene expression and the inhibitory effect was not a virus-specific phenomenon.

LMP-1-mediated Inhibition of VSV Replication Is Associated with ISG Induction—In order to examine the relationship between the inhibition of viral protein expression and induction of ISGs by LMP-1, we tested if LMP-DM mutant that failed to induce any ISGs could inhibit VSV protein expression. As shown in Figure 3A, LMP-1, but not LMP-DM, inhibited VSV protein expression. These data suggest

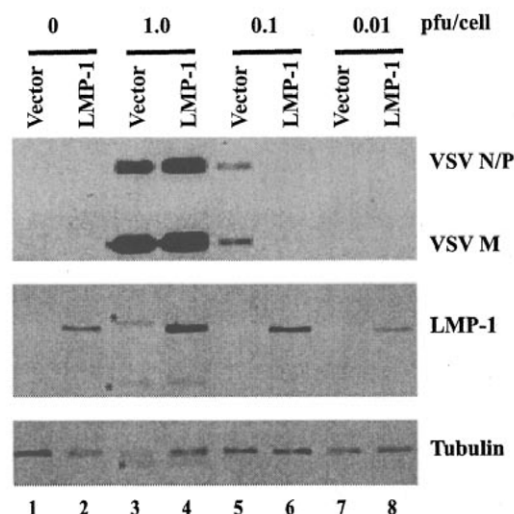


Figure 2. LMP-1 inhibits VSV protein expression. DG75 cells were transfected with pcDNA3 (vector, lanes 1, 3, 5, and 7) or LMP-1 expression plasmids (lanes 2, 4, 6, and 8). The transfected cells were isolated and infected with VSV for 8–10 h. Cell lysates were used for Western blot analysis using VSV-specific antibodies. The same membrane was stripped and used with different antibodies. The multiplicity of infection is shown on the top. The identity of the proteins is as shown. * = residual VSV proteins.

that LMP-1 is able to inhibit VSV gene expression and the inhibition is associated with the induction of antiviral ISGs.

In addition to viral protein expression, we also performed plaque assays to detect VSV production in the media. In agreement with protein expression data (Figs. 2 and 3A), LMP-1 was able to inhibit the production of VSV, up to 100-fold (Figure 3B). Thus, data from both viral protein expression and virus production suggested that LMP-1 inhibited the replication of VSV. Furthermore, marginal inhibition of VSV production was observed with the LMP-DM mutant (Figure 3B). Because LMP-DM failed to induce any ISGs tested, these data suggest that induction of ISGs is an important step in the establishment of antiviral state by LMP-1.

Cells Harboring EBV Inhibited VSV Replication—It is important to determine whether LMP-1 in native environment in EBV latency cells also induces the expression of ISGs and inhibits viral replica-

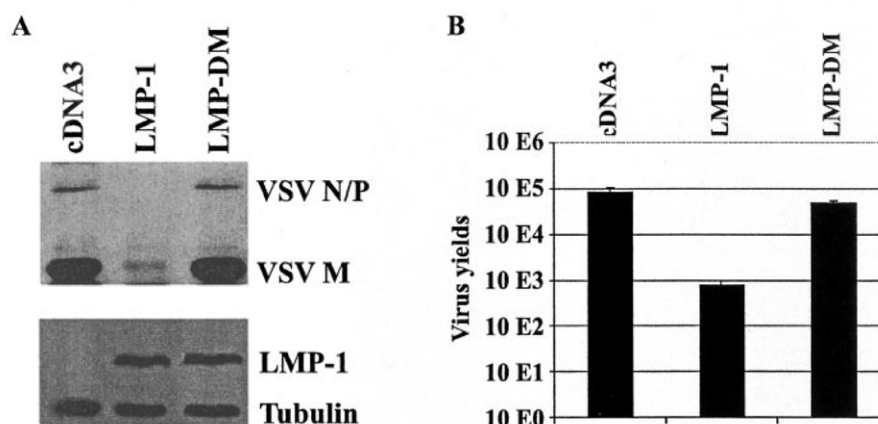


Figure 3. LMP-1 inhibits VSV virus production. A, LMP-1 mutant fails to inhibit VSV protein expression. DG75 cells were transfected with pcDNA3, LMP-1, or LMP-DM expression plasmids. The transfected cells were isolated and infected with VSV at 0.1 moi for 8–10 h. Cell lysates were used for Western blot analysis using VSV-specific antibodies. The same lysates were used for detection of tubulin and LMP-1 antibodies. The identity of proteins is as shown. B, LMP-1 inhibits VSV production. DG75 cells transfected with pcDNA3, LMP-1, or LMP-DM were infected by VSV at 0.1 moi for 12 h. VSV production was measured by plaque assay. The virus yields are as shown in logarithm units. The results represent an average of three independent experiments. Error bars represent S.D.

tion. BL41, a Burkitt lymphoma line, and its EBV-infected derivative, BL41-EBV, were used to investigate the issue. The two cell lines have the identical genetic backgrounds and have been shown to be a good pair to analyze the effect of EBV on the cellular gene expression (35). BL41-EBV is a type III latency cell line in which LMP-1 is expressed (Figure 4C). Total RNA or cell lysates were prepared from these cells. RPA experiments demonstrated that expression of OAS and STAF-50 were increased in cells harboring EBV (Figure 4A). Also, Western blotting demonstrated a clear increase in both ISG15 and STAT-1 proteins expression after EBV infection (Figure 4B). In addition, IRF-7 is also highly expressed in BL41-EBV (26). These data suggest that the induction of antiviral ISGs is a consequence of the establishment of EBV latency in human B cells.

Next, we examined the viral replication efficiencies in BL41 and BL41-EBV cell lines. As shown in Figure 4C, VSV protein expression was significantly lower in BL41-EBV in which LMP-1 is expressed. Also, VSV viral productions were consistently lower in the BL41-EBV cells (Figure 4D). Statistically, the differences in viral yields between BL41 and BL41-EBV are highly significant ($p < 0.01$). The modest inhibition of viral replication may be caused by other EBV latent gene(s) has ability to neutralize the effect of LMP-1. These data suggest that EBV latency cells have the ability to suppress viral replication, and LMP-1 in native environments may be capable of inhibiting viral replication.

IFNs Were Not Involved in LMP-1-mediated Antiviral Effect in DG75 Cells—One possible mechanism for LMP-1 to induce the expression of ISGs and antiviral state is indirectly via the induction of IFNs. The possibility was examined by several approaches in DG75 cells in which the induction of ISGs and antiviral effect of LMP-1 were observed (Figs. 1, 2, 3). First, we examined if LMP-1 induced the synthesis of IFN RNAs in LMP-1 expressing cells. RT-PCR was carried out in LMP-1-transfected cells with IFN and actin primers. Two sets of IFN primers were used in this assay. One was designed to detect mRNAs of all IFN- α subtypes (especially IFN- α 1, -2, -4, -7, -10, -13, and -14) and another to detect only IFN- α 1 mRNA, the most abundant IFN- α subtype (36, 37). As shown in Figure 5A, RT-PCR results suggested that all IFN- α subtypes and IFN- α 1 were not synthesized in greater amount in LMP-1-expressing cells; suggesting that LMP-1 might not be able to induce the expression of IFN- α .

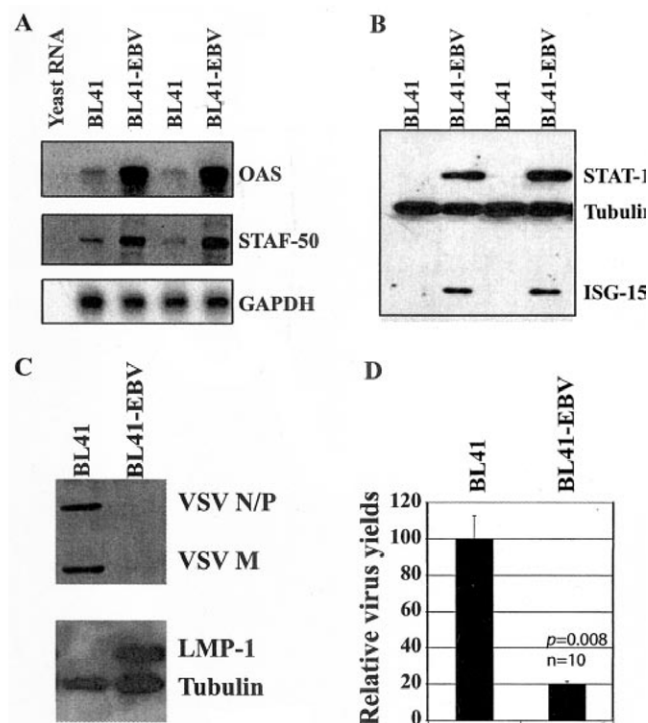


Figure 4. EBV latency has antiviral property. A, RNA expression of OAS and STAF-50 are associated with EBV type III latency. Total RNAs (5 μ g) from BL41 or its EBV-infected counterpart BL41-EBV were used for RPA with mixed probes of OAS, STAF-50, and GAPDH. Yeast RNA was used as negative control. Specific protections of OAS, STAF-50, and GAPDH RNAs are indicated. B, expression of ISG15 and STAT-1 proteins are associated with EBV type III latency. Lysates from BL41 and BL41-EBV were used for Western blot analysis with tubulin, ISG15 as well as STAT-1 antibodies. The identity of proteins is as shown. C, VSV protein expression is inhibited in EBV latency. BL41 and BL41-EBV were infected with VSV at 0.1 moi for 8–10 h. Cell lysates were used for Western blot analysis using VSV, LMP-1, and tubulin antibodies. The identity of proteins is as shown. D, VSV production is inhibited in EBV latency. VSV (0.1 moi) was used to infect BL41 and BL41-EBV. Relative VSV productions in BL41 and BL41-EBV after 12 h post-infection are shown. The significance in VSV yields between BL41 and BL41-EBV was calculated by Mann Whitney U Test with the use of the Statistica 6.0 program. p value and sample numbers (n) are shown.

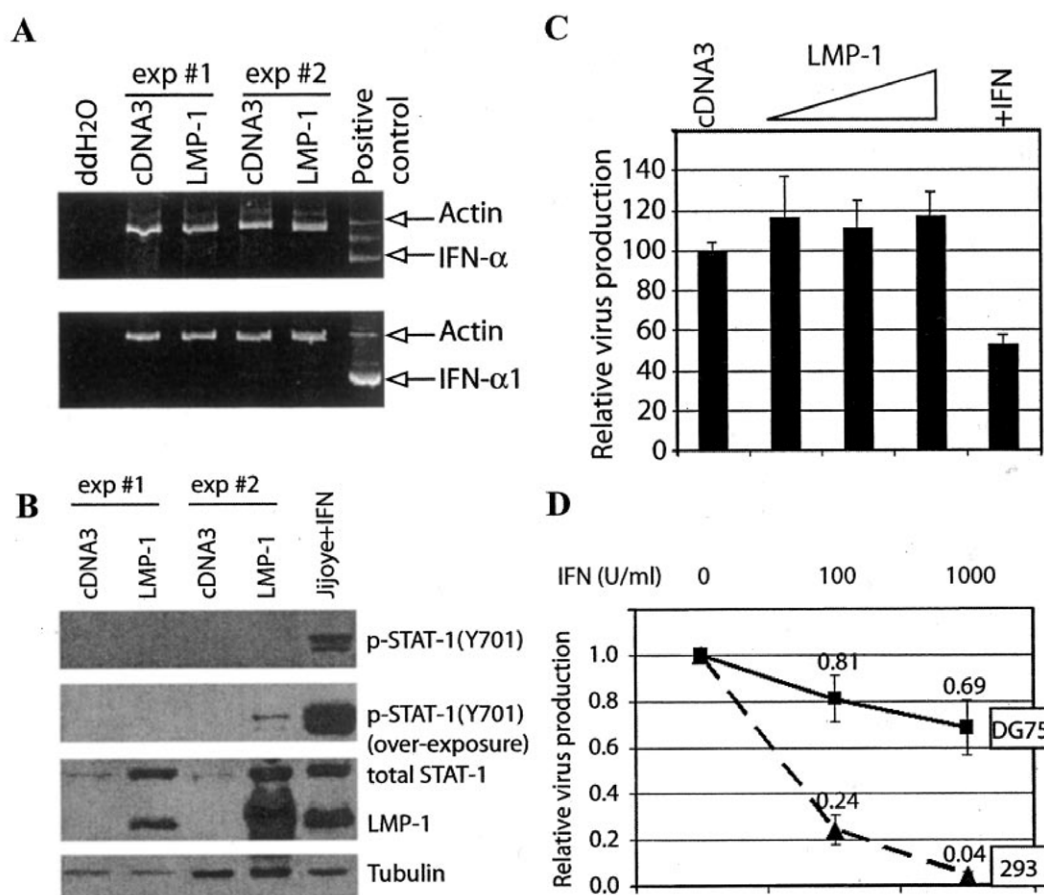


Figure 5. IFNs were not involved in LMP-1-mediated antiviral effect in DG75 cells. *A*, LMP-1 did not induce great amount of IFNs. IFN- α mRNA levels were analyzed in cells expressing LMP-1 and its vector controls. RNA from 293 cells transfected with IRF7 Δ 247–467 was used as positive control. One-step RT-PCRs were performed with consensus IFN- α and actin primers or specific IFN- α 1 primers plus actin, respectively. The PCR products were separated on 8% polyacrylamide gel. The identity of the RT-PCR products is as shown. *B*, LMP-1 did not induce the phosphorylation of STAT-1 at a critical tyrosine residue. Western blot with phosphospecific STAT-1 antibody was first performed. The membranes were then stripped and antibodies against intact STAT-1, LMP-1, as well as tubulin were used to determine the expression of total STAT-1 and others. The positive control was lysates from Jijoye cells that had been treated with IFN- α for 30 min. The identity of proteins is as shown. *C*, media from LMP-1-expressing cells were unable to induce an antiviral state. Media from LMP-1-expressing cells were obtained by removal of cells by centrifuging. The media were mixed with fresh DG75 cells at 1:1 ratio for 12 h before infection of VSV at 0.1 moi. The positive control was IFN-treated DG75 cells. The relative VSV production is as shown. *D*, VSV production is insensitive to IFN in DG75 cells. 293 and DG75 cells were treated with IFN overnight, and then infected with VSV (0.1 moi) for 12 h. The relative VSV production is as shown. Squares, DG75; triangles, 293 cells.

Our data in Figure 5A could not rule out the possibility that LMP-1 might induce a rare member of IFN family, which might be responsible for the induction of ISG. We reasoned if LMP-1 induced the expression of biologically active IFNs, STAT-1 would be activated by these IFNs. It is well known that the phosphorylation at the critical Tyr-701 residue of STAT-1 is a crucial event for its function in IFN signaling. We tested the activation status of STAT-1 by the use of specific antibodies for phospho-STAT-1 (Tyr-701) in LMP-1-expressing cells. As shown in Figure 5B, STAT-1 was not phosphorylated, or very marginally activated, at the critical Tyr-701 residue in LMP-1-positive cells. Also, STAT-1 in LMP-1-positive EBV latency cells is not activated (25). Thus, the data suggest that LMP-1 did not produce significant amount of biologically active IFNs that were capable of activating STAT-1.

If LMP-1 could induce IFNs and/or other soluble antiviral proteins, then the media from LMP-1-expressing cells would be able to induce an antiviral state on other cells. To test this possibility, the conditional media from LMP-1-expressing cells were used to treat DG75 cells overnight and these cells were then infected with VSV. As shown in Figure 5C, the conditional media failed to inhibit VSV replication in DG75 cells, but treatment of IFN marginally interfered

with VSV production. The results suggest the media from LMP-1-expressing cells could not induce an antiviral state (Figure 5C). Thus, LMP-1-mediated antiviral response is not related to secreted IFNs or other antiviral factors.

VSV Production Is Insensitive to IFN in DG75 Cells—It is interesting that IFN-treated DG75 cells were still supporting decent VSV production (Figure 5C). We suspected that DG75 cells might be insensitive to IFN-mediated inhibition of VSV production. Both 293 and DG75 cells were treated with the same IFN for same time, and infected with VSV. As shown in Figure 5D, whereas IFN significantly inhibited VSV production in 293 cells, IFN could not produce a significant inhibition of VSV production in DG75 cells. Thus, VSV production is insensitive to IFN treatment in DG75 cells. It is of note that DG75 cells are responsive to IFN treatment: IRF-7, ISG15, and PKR were increased upon treatment of IFNs (data not shown). Because our data show that LMP-1 significantly inhibit VSV production in the same cells (Figure 3), these results strongly suggest that LMP-1-mediated antiviral response in DG 75 cells is irrelevant to IFN production.

Viral Infection Induced the Expression of LMP-1—Because the LMP-1 promoter region has an ISRE-like element and is responsive

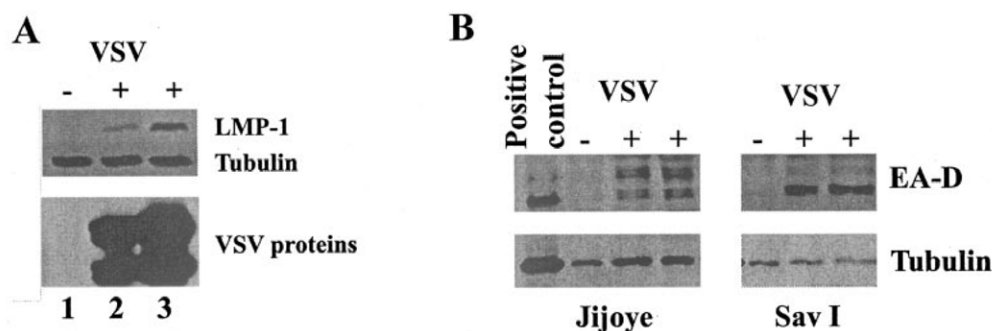


Figure 6. Responses of EBV latency cells to VSV superinfection. A, superinfection of EBV latency cells induces LMP-1 expression. VSV was used to infect P3HR1 cells for 16–24 h. Western blot with LMP-1 and tubulin antibodies was performed first. The membrane was then stripped and probed with VSV antibody. The identity of proteins is as shown. Lane 1, uninfected cells; lanes 2 and 3, cells infected with VSV at 0.1 or 1 moi, respectively. B, VSV infection induces EA-D expression. VSV was used to infect Sav I and Jijoye cells for 24 h. Cell lysates were separated by SDS-PAGE. Western blot with EA-D antibody was performed first. The membrane was then stripped and probed with tubulin antibody. The positive controls are EBV-BZLF1 transfected cells. The identity of proteins is as shown.

to IRF-7 (38), we reasoned that LMP-1 might be inducible by viral superinfection in EBV latency cells. P3HR1 is a Burkitt's lymphoma line that lacks the *EBNA-2* gene and a portion of *EBNA-LP* (31). As a result of the deletion, P3HR1 cells do not express *EBNA-2*, and consequently because *EBNA-2* *trans*-activates the *LMP-1* promoter (39–42), express a very low level of LMP-1. As shown in Figure 6A, VSV infection induced expression of LMP-1 protein in P3HR1 cells. Similar results were obtained in Daudi cells and sendai virus also induced the expression of LMP-1 (data not shown). However, expression of LMP-1 RNA was sensitive to protein synthesis inhibitor, cycloheximide (data not shown). In addition, VSV marginally activated *LMP-1* promoter reporter constructs in EBV-negative cells (data not shown). Thus, these data suggest that VSV infection triggers the synthesis of LMP-1, but *de novo* protein synthesis of viral or cellular factor(s) is required for the induction.

Viral Infection Induced Lytic Replication of EBV—To test what may happen in EBV latency cells super-infected with other viruses, we examined if VSV could disrupt EBV latency by inducing lytic replication of EBV. We used EBV EA-D (BMRF-1) expression as a marker for lytic replication. The essential function of EA-D in EBV lytic replication has been well established and using EA-D as an indicator of lytic replication has been appreciated in the field for years (43–49). As shown in Figure 6B, VSV infection induced expression of EA-D in two latency cell lines. Multiple bands for EA-D are a common phenomenon due to the phosphorylation (48, 49). Sendai virus infection also induced the expression of EA-D (data not shown). These data strongly suggest that viral superinfection might disrupt EBV latency by inducing EBV lytic replication.

Discussion

It is well established that OAS is a major component of cellular antiviral responses (11, 12); IRF-7 is a key factor responsible for IFN production upon viral infection (13–16); and STAT-1 is an essential transducer for IFN signal transduction (50, 51). These genes are essential for cells to establish an antiviral state and play important roles in viral pathogenesis (11, 12). In this report, we have shown that LMP-1 induces several antiviral ISGs including OAS and provided evidence that LMP-1 has antiviral effect. In addition, we demonstrate that EBV type III latency cells, in which LMP-1 is expressed at physiological levels, have the capability to induce ISG productions and inhibit superinfecting virus replication.

The induction of ISGs may be the molecular bases for the antiviral effect of LMP-1. Some ISGs, such as OAS, when expressed at

high levels are sufficient to repress viral replication in the absence of other ISGs (18). Overexpression of IRF-7 alone is also able to inhibit VSV replication as expected (data not shown). The inhibition is apparently related to the induction of IFN because an IRF-7 mutant that cannot induce IFN production failed to inhibit the replication of VSV (data not shown). In addition, LMP-1 may modulate some antiviral protein activity. The transmembrane domain of LMP-1 has been shown to initiate phosphorylation of eIF-2 α , which would result in inhibition of viral replication (11, 12, 52). Thus, through induction of antiviral ISGs and modulation of existing protein activities, LMP-1 may obtain the ability to suppress viral replications. In support of such a notion, LMP-DM, which failed to induce the expression of several ISGs, was unable to induce antiviral state (Figure 3). However, the major contributor(s) in LMP-1-mediated antiviral response is currently unknown.

The induction of antiviral OAS, STAF-50, STAT-1, and ISG-15 is associated with EBV latency and LMP-1 expression (Figure 4). In addition, IRF-7 is also highly expressed in EBV latency (26, 53), and induction of other ISGs in EBV latency has been reported (35). These results suggest that the induction of ISGs is likely to be a property of EBV latency and, based on our results, related to the expression of LMP-1. Furthermore, EBV latency cells do have antiviral effect (Figure 4, C and D). The antiviral effect is modest but statistically highly significant ($p < 0.01$). The modest antiviral effect in EBV latency cells might be due to other viral factor(s) diminishing the effect of LMP-1. *EBNA-2* has been shown to block the function of ISGs (54). Also, other herpesviruses, such as herpes simplex virus 1 (HSV-1) and cytomegalovirus (CMV), initiate an ISG production and use other viral genes to block antiviral effects (55–62).

Because ISG induction is a major outcome with IFN treatment, one possibility is that the activation of ISGs by LMP-1 is indirect via the induction of IFNs. However, it is unlikely that IFN is a major player in the process. First, RT-PCR results suggest that IFNs were not synthesized in great amount in LMP-1-expressing cells (Figure 5A). Second, LMP-1-induced expression of STAT-1 was not phosphorylated at the tyrosine residue 701, the phosphorylation of which is critical for IFN signaling (Figure 5B). Third, the media from LMP-1-expressing cells could not induce an antiviral state on other cells (Figure 5C). Fourth, DG75 cells are insensitive to IFN-induced inhibition of VSV production (Figure 5D). Fifth, LMP-1 selectively induces ISGs (Figure 1), and LMP-1 can induce phosphorylation of eIF-2 α , apparently not through PKR (52). Sixth, the induction of ISGs in EBV latency was shown to be independent of IFNs (35). Seventh and finally, IFN was not involved in the induction of ISGs upon

infection by other herpesviruses (35, 60, 61, 63, 64). Thus, based on our data presented here and previously published results, it is likely that the observed antiviral function is a direct effect of LMP-1, and IFN may not be a major factor in the process at least in DG75 cells.

LMP-1 may use multiple mechanisms for the induction of ISGs. Notably, LMP-1 induces both STAT-1 and -2 in Akata cells; however, LMP-1 cannot induce IRF-7 in the same line (25, 26). Furthermore, the detailed domain requirement for the induction of IRF-7 and STAT-1 are different (25, 27). Thus, inductions of IRF-7 and STAT-1 by LMP-1 are obviously via different mechanisms. We also tested the role of LMP-1 in the activation of IRF family members. Specifically, LMP-1 could not induce the expression of IRF-1 and IRF-3 in DG75 cells. In addition, LMP-1 failed to activate IRF-3 based on the subcellular localization (data not shown). Thus, IRF-7, which can be induced and activated by LMP-1, might be the most relevant member in IRF family to induce ISGs. All ISG promoter regions have one or more ISREs (11, 12). Activated IRF-7 might be able to selectively induce the expression of ISGs based on the binding affinities to various ISREs and other factors. The expression of Tap-2, another ISG, was shown to be regulated by IRF-7 (34). Although LMP-1 cannot activate STAT-1 protein at the critical tyrosine residue, but because STAT-1 can also induce ISGs under non-activated state (65), we suspect that STAT-1 may also be involved in the induction of certain ISGs in the absence of IFN.

EBV has both latency and lytic replication in its life cycle. EBV latency can be disrupted by lytic replication, which can be induced by many chemical or physiological factors. In this report, we have shown that the infection of EBV latency cells by VSV or Sendai virus induced EBV lytic replication (Figure 6 and data not shown). In retrospect, EBV superinfected EBV latency cells induced lytic replication (66–69). In addition, HHV-6 and CMV infection of EBV latency cells would also lead to lytic replication of EBV (70–72). These reports plus our data in Figure 6 have suggested a general conclusion that a viral superinfection of EBV latency cells would lead to lytic replication of EBV and the disruption of EBV latency program.

EBV has several latencies with various expression levels of LMP-1. A superinfection of EBV latent cells by a foreign virus may trigger the synthesis of LMP-1 (Figure 6A). Interestingly, infection of EBV latency cells by EBV itself or HHV-6 also induced the expression of LMP-1 (73–75). These data suggest that LMP-1 induction may be a common response of EBV latency cells to superinfection, and the antiviral effect of LMP-1 may be a general property of all EBV latencies, regardless of the original LMP-1 status. Based on facts that induction of LMP-1 RNA was sensitive to protein synthesis inhibitor (data not shown), and fact the LMP-1 is expressed in lytic replication cycle (73, 76, 77), it is likely the induction of EBV lytic replication has resulted in the synthesis of LMP-1. Because LMP-1 is able to inhibit EBV lytic replication via at least two mechanisms (78, 79), the antiviral function of LMP-1 might also be functioning in lytic replication cycle by inhibiting EBV lytic replication.

Finally, it is of note that an antiviral stage can be induced by many different stimuli; such as bacterial infection and environmental stresses (11, 12). Thus, the establishment of an antiviral stage is not solely for the protection of the cells from super infection, but a general response to protect the cells in harsh environments. The antiviral function of LMP-1 may help EBV latency cells to cope with a variety of foreign attacks as well as changes in cellular environments by promoting EBV latency as well as blocking EBV lytic replication.

In summary, our results indicate that LMP-1 induces antiviral ISGs selectively, and has an antiviral effect. LMP-1-mediated antiviral effect may be an intrinsic part of EBV latency program, and may assist the establishment and/or maintenance of EBV latency.

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